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Isolation of a dual plant promoter fragment from the Ti plasmid of *Agrobacterium tumefaciens*

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The two most abundant transcripts derived from TR-DNA within plant cells transformed by an octopine strain of *Agrobacterium tumefaciens* arise from divergent transcription, both originating within an ~500 bp section of the T-DNA. Using a combination of subcloning and exonuclease digestion, a 479-bp DNA fragment, directly flanked by the initiation codons for the two adjacent open reading frames, was isolated. The resulting DNA fragment was fused, in both orientations, to the neomycin phosphotransferase (NPT II) gene of the transposon Tn5 prior to introduction into *Nicotiana tabacum* cells *via* the Ti plasmid. The intergenic fragment was found to initiate expression of the NPT II gene in either orientation as assayed by kanamycin resistance of the transformed plant tissue as well as by enzymatic assay of the NPT II gene product. The plasmids described here are potential selection-expression vectors for plant systems.

Key words: Agrobacterium tumefaciens/antibiotic resistance/gene expression vector/promoter regions/selectable marker genes

Introduction

Crown gall disease, the unusual symbiotic relationship between the common soil bacteria, *Agrobacterium tumefaciens*, and a broad range of dicotyledonous plants, has proven to be a unique and valuable natural system for the transfer of foreign DNA into plants (for recent review, see Zambryski *et al.* 1984). The transferred DNA (T-DNA) originates within the Ti plasmid of the bacterium and is eventually stably integrated into the chromosomal DNA of the host plant (Chilton *et al.*, 1977, 1980; Schell *et al.*, 1979). The T-DNA is subsequently expressed, resulting in undifferentiated, axenic growth of the plant cells (Braun, 1956) and the production, by the transformed tissue, of a class of unusual chemical compounds called opines (Bomhoff *et al.*, 1976; Tempé *et al.*, 1980).

Analysis of transcription within transformed plant tissue and isolated nuclei has revealed that T-DNA transcripts are initiated by RNA polymerase II (Willmitzer *et al.*, 1981b) and that the relative steady-state concentrations of the different T-DNA mRNA species vary significantly (Drummond *et al.*, 1977; Willmitzer *et al.*, 1981a; Gelvin *et al.*, 1981). The most extensively studied T-DNA genes are those coding for the enzymes involved in opine biosynthesis: nopaline synthase (NOS) (De Greve *et al.*, 1982) and octopine synthase (OCS) (Depicker *et al.*, 1982). Despite the prokaryotic origin of these genes, both contain, at the appropriate locations, strings of bases matching the eukaryotic consensus sequences for both initiation and termination of transcription. The DNA sequences containing transcriptional control signals for the NOS gene have been further defined by fusion to the coding regions of several prokaryotic genes. These chimaeric genes, after introduction into plant cells using Ti plasmid derived vectors, were expressed, producing the appropriate functional enzymes (Herrera-Estrella *et al.*, 1983a, 1983b; Bevan *et al.*, 1983; Fraley *et al.*, 1983).

Research into T-DNA transfer, integration and expression was initially focused upon the T-DNA responsible for neoplastic transformation of the plant cell. In octopine type Ti plasmids, these oncogenic functions, along with the OCS gene, are located within the left transferred segment (TL). Octopine Ti plasmids, however, also contain a second section of T-DNA, TR(ight), which is generally independently integrated into the plant genome (Thomashow *et al.*, 1980; De Beuckeleer *et al.*, 1981). The TR-DNA is not required for tumor formation but has been associated with the production of the agropine family of opines (Velten *et al.*, 1983; Salomon *et al.*, 1984; Ellis *et al.*, 1984). Recently, plant tumor lines producing both octopine and agropine were found to contain five transcripts which map to the TR-DNA (Velten *et al.*, 1983; Winter *et al.*, 1984; Karcher *et al.*, 1984). Of the five TR transcripts, three, including the two most abundant mRNAs (1′ and 2′) have been linked with agropine production (Salomon *et al.*, 1984; Ellis *et al.*, 1984).

The 1′ (1.6 kb) and 2′ (1.45 kb) transcripts (Velten *et al.*, 1983), coding for, respectively, the second and first enzymes in the agropine biosynthetic pathway (Ellis *et al.*, 1984), show steady-state levels significantly greater than that of the NOS transcript, previously the most abundant T-DNA transcript (L. Willmitzer, personal communication). The two mRNAs are divergently transcribed and both originate within a 500-bp DNA segment of the TR-DNA (Velten *et al.*, 1983; Winter *et al.*, 1984; Karcher *et al.*, 1984). DNA sequence analysis of the promoter region has shown that there are only 482 bp of intergenic DNA between two divergent open reading frames corresponding to the 1′ and 2′ transcripts [our data (Figure 6) and Barker *et al.*, 1983]. The length of the intergenic DNA corresponds roughly to twice the reported length of the OCS and NOS promoters (~200–300 bp from the coding region, Koncz *et al.*, 1983), supporting the possibility that two divergent promoters are contained within the intergenic DNA.

In order to determine if the 1′–2′ intergenic region actually contains all DNA signal sequences necessary to initiate transcription in both directions, the intergenic fragment was isolated and fused, in both orientations, to the neomycin phosphotransferase II (NPT II) gene of Tn5.

Results

Isolation of the 1′–2′ promoter region

A 676-bp *Alu*I restriction fragment, containing the entire 1′–2′ intergenic region of the pTiAch5 plasmid, was cloned into the *Sma*I site of pUC8 (Messing and Vieira, 1982) (Figure 1). The resulting plasmid, pAR1200, contains a unique *Cla*I

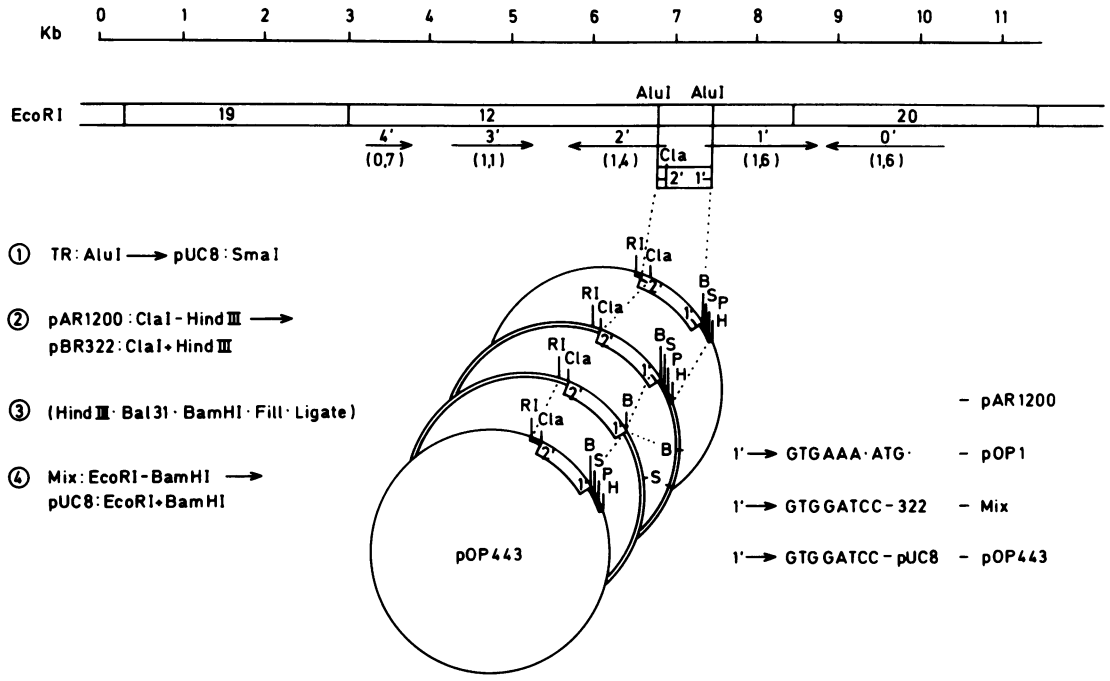

Fig. 1. Isolation of the 1′−2′ promoters. The isolation and cloning of the 1′−2′ promoter fragment (given in abbreviated form at the left) has been described in the Results section. The transcription map of the TR region, indicating the origin of the 1′−2′ promoter fragment, is shown at the top. The transcript sizes are indicated, in kilobases, below each arrow. The abbrevations for restriction enzyme cut sites are 'RI' = *Eco*RI, 'Cla' = *Cla*I, 'B' = *Bam*HI, 'S' = *Sal*I, 'P' = *Pst*I and 'H' = *Hind*III. The DNA sequence in the region of the 1′ initiation codon is shown to the right of the appropriate plasmid construction.

restriction site which cuts directly at the initiation codon of the 2′ open reading frame (ATCG:ATG:GAC: → 2′). To remove the remaining 2′ coding sequence, including the initiation codon, the *Cla*I to *Hind*III (from the pUC8 polylinker) fragment was recloned into pBR322 between the corresponding plasmid restriction sites, producing pOP1. Further manipulation of pOP1 was required to remove residual 1′ coding sequences as well as prepare a suitable restriction site near the 5′ end of the 1′ open reading frame. Following cleavage by *Hind*III, pOP1 was digested with exonuclease *Bal*31, cut with *Bam*HI, DNA ends filled-in with large fragment DNA polymerase I and the plasmid recircularized. Ligation of a terminal G residue from the exonuclease digestion to the filled-in *Bam*HI cut site regenerates the *Bam*HI restriction site (Figure 1). After digestion of the resulting mixture of plasmids with *Eco*RI and *Bam*HI, a DNA fragment of the expected size was isolated by gel electrophoresis and recloned into the pUC8 vector (Figure 1). DNA sequence analysis of the resulting plasmid (data not shown) indicated that the *Bam*HI restriction site produced at the 1′ end of the 1′−2′ intergenic region is located 3 bp upstream of the 1′ start codon (see Figure 1).

Fusion of the 1′ and 2′ promoters to the NPT II gene

The plasmid pLGV1103 (Herrera-Estrella *et al.*, in preparation) was used as a vector for the fusion of both 1′ and 2′ promoters to the NPT II gene. This plasmid contains the NPT II gene of Tn5 flanked, at the 5′ end, by the NOS promoter and, at the 3′ end, the polyadenylation site of the OCS gene (De Greve *et al.*, 1982). pLGV1103 also contains a second Km resistance gene (APH type I), derived from Tn903, under control of the original prokaryotic promoter. The Tn903 derived Km resistance gene is not homologous to the NPT II of Tn5 and is used for co-integrate selection after mobilization to *A. tumefaciens*. The NOS promoter in pLGV1103 is bordered by *Eco*RI and *Bcl*I restriction sites, the latter directly adjacent to the initiation codon of the NPT II gene (Herrera-Estrella *et al.*, in preparation). The 1′−2′ promoter fragment of pOP443 was, therefore, further manipulated to provide restriction sites complementary to those in pLGV1103, allowing direct replacement of the NOS promoter with the 1′−2′ promoters.

The TR promoter fragment of pOP443 contains, at the 2′ end, a small *Eco*RI to *Cla*I DNA fragment derived from pBR322. This sequence contains a possible translational initiation codon between the 2′ promoter and the *Eco*RI site, potentially reducing translational efficiency of any genes fused to the 2′ promoter at the *Eco*RI site (Kozak, 1984; Liu *et al.*, 1984). In order to make a useful cloning site directly downstream of the 2′ promoter, the DNA sequence between the *Eco*RI and *Cla*I restriction sites was deleted prior to fusion of the 1′ promoter to the NPT II gene. The deletion was produced by digestion with *Eco*RI and *Cla*I, followed by polymerase I filling of the staggered ends and blunt end ligation. This protocol produces, in the plasmid pOP4434, an *Eco*RI restriction site at the position of the original 2′ start codon (Figure 3). The 1′−2′ promoter fragment of pOP4434, flanked directly by *Eco*RI (2′ end) and *Bam*HI (1′ end), was then inserted into pLGV1103 in place of the NOS promoter fragment (*Eco*RI to *Bcl*I, Figure 2). Ligation of the complementary *Bam*HI (1′ end of 1′−2′ fragment) and *Bcl*I (5′ end of the NPT II gene) staggered ends produced the 1′-NPT II chimaeric gene in plasmid pAK1003.

As a control against either transcriptional read-through from the vector, or the fortuitous generation of a plant promoter at the vector-insert junction opposite the NPT II gene, a 2.2-kb DNA fragment containing the chloramphenicol resistance determinant of pBR325 was inserted at the *Eco*RI

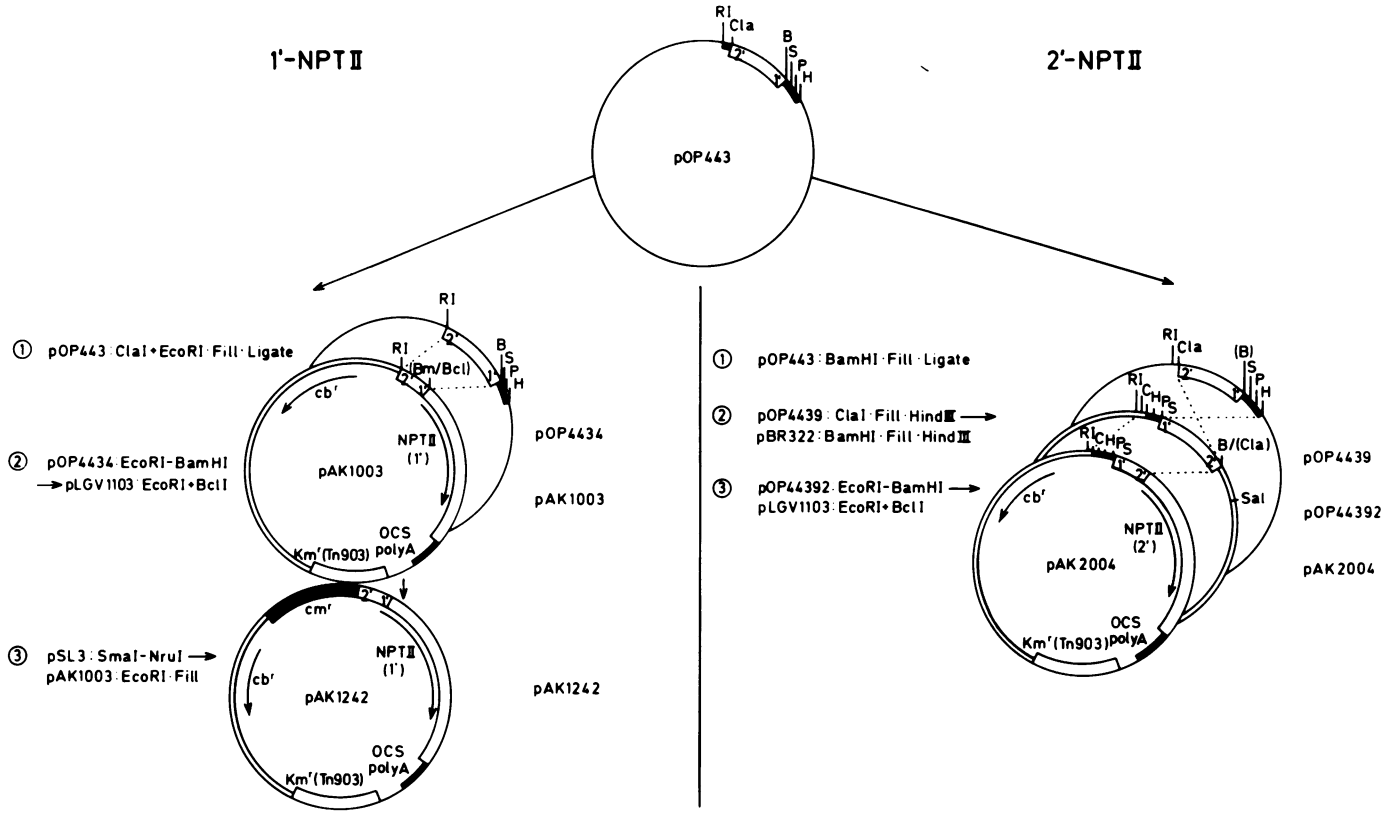

Fig. 2. Fusion of the 1′ and 2′ promoters to the NPT II gene. Fusion of both the 1′ and 2′ promoters to the NPTII gene has been described in the Results section. Short descriptions of the procedures involved are listed to the left of the plasmid maps. Restriction site abbrevations are as in Figure 1 with the addition of 'Bcl' for *Bcl*I and 'C' for *Cla*I. Other abbreviations are: 'cb r' for carbenicillin resistance; 'km r (Tn903)' for bacterial kanamycin resistance derived from Tn903; 'OCS poly(A)' for octopine synthase polyadenylation control signal. Restriction sites in brackets indicate cleavage sites lost after heterologous ligations.

```
.pAK1003
          EcoRI
(pBR322) GAATTCGATTTGGTGTATCG <- 2'== 1'-> CACGTGGATC:ATG:ATT:GAA:(NPT II)
======== :CAT:CGATTTGGTGTATCG <- 2'== 1'-> CACGTGAAA:ATG:CCA:ATT: (gp 1')

pAK2004
EcoRI       ClaI  HindIII PstI  SalI
GAATTC(322)ATCGATAAGCTTGGCTGCAGGTCGACGGATCGA <- 1'== 2'-> TCGGATC:ATG:(NPT II)
=====================================:CAT:T <- 1'== 2'-> TCG:ATG:GAC:(gp 2')
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Fig. 3. DNA sequence at the promoter-gene junctions. The predicted DNA sequence at the promoter-NPT II and promoter-vector junctions of the 1′ (pAK1003) and 2′ (pAK2004) are shown. Indicated above the sequences are the start codon for the NPT II gene (half arrow) and potential cloning sites downstream of the second promoter. The lower sequence of each plasmid indicates the relative position of the 1′ and 2′ initiation codons (indicated by half arrows) from the original TR genes. Between the *Eco*RI and *Cla*I sites of pAK2004 are 19 bp (not listed) derived from pBR322.

site of pAK1003 (Figure 2). The resulting plasmid, pAK1242, was subsequently transformed into tobacco and NPT II expression compared with that from pAK1003.

Fusion of the 2′ promoter to the NPT II gene required two further manipulations of the dual promoter fragment of pOP443. Initially, the *Bam*HI site of pOP443 was destroyed by filling-in the cut plasmid with polymerase I and recircularization. This plasmid, pOP4439, was then cut with *Cla*I, the staggered ends filled-in the DNA polymerase I, and digested with *Hind*III. The resulting fragment, containing the 1′ and 2′ promoters, was inserted into pBR322 between the *Hind*III site and the previously filled-in *Bam*HI site. The blunt-end ligation of filled-in *Cla*I and *Bam*HI sites regenerated the *Bam*HI site, producing the plasmid pOP44392 (Figure 2). Replacement of the *Eco*RI to *Bcl*I fragment of pLGV1103 with the *Eco*RI to *Bam*HI fragment of pOP4439 fused the 2′ promoter to the coding region of the NPT II gene. In addition to the 2′-NPT II chimaeric gene, the resulting plasmid, pAK2004, contains several convenient restriction sites, derived from the polylinker of pUC8, immediately downstream from the 1′ promoter (Figure 2).

The predicted DNA sequence of the various promoter junctions are given in Figure 3. In both NPT II chimaeric genes, the first initiation codon begins the coding sequence of

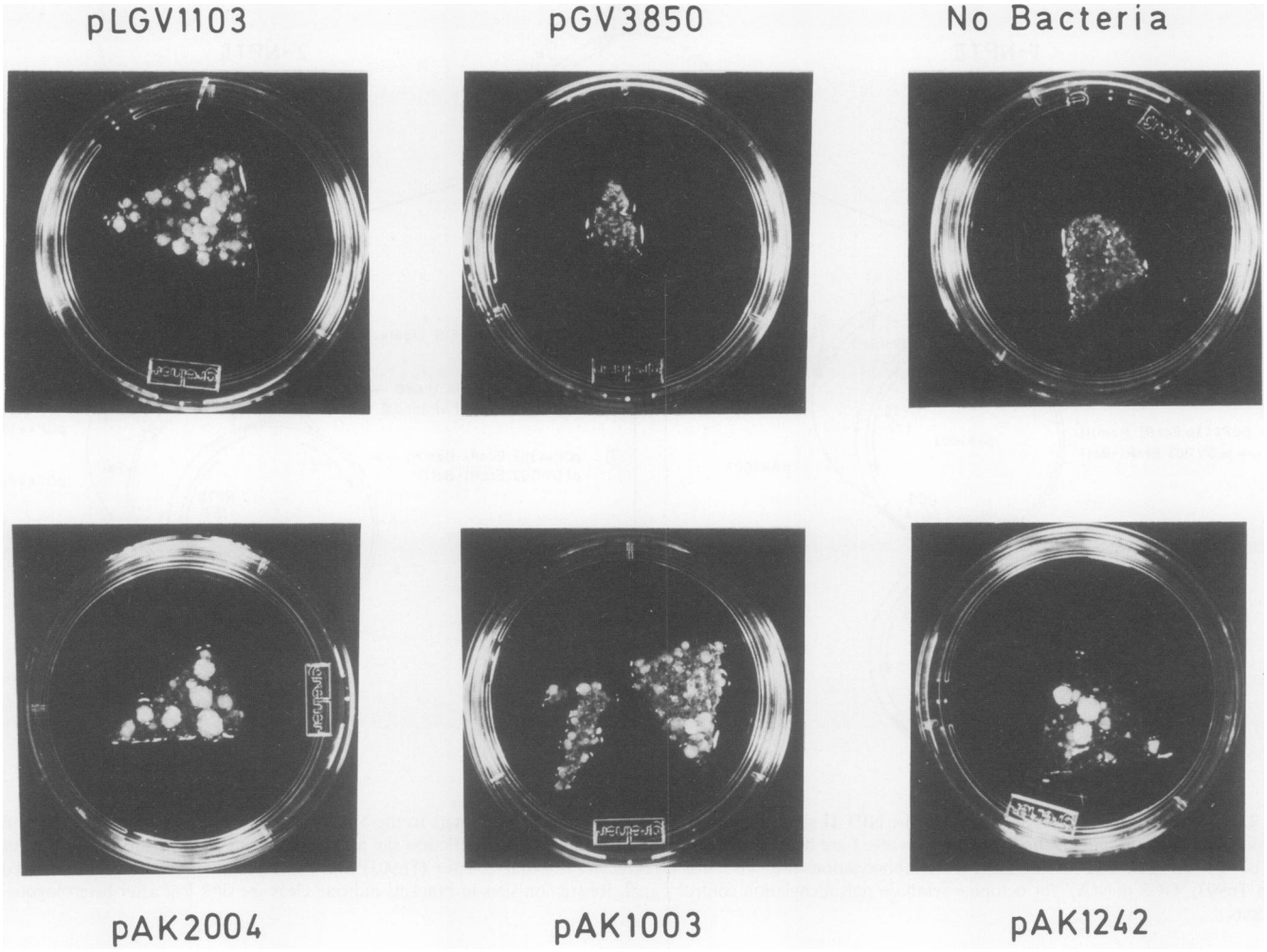

Fig. 4. Selection of transformed calli for Km resistance. Protoplasts transformed with the indicated constructions were grown under 1000 μg/ml for 4–6 weeks (see Materials and methods). Negative controls were protoplasts cultured without *Agrobacteria* (no bacteria) and protoplasts co-cultivated with *Agrobacteria* containing no NPT II construction (pGV3850). The positive control was the plasmid pLGV1103 (NOS promoter fused to the NPT II gene) co-integrated into pGV3850.

the NPT II gene and is within 3 bp of the position, relative to the promoter, of the start codon of the original TR gene (see Figure 3).

Introduction of the 1' and 2' promoter-NPT II fusions into tobacco cells

The disarmed Ti plasmid, pGV3850, developed by Zambryski *et al.* (1983) was used to transfer the chimaeric NPT II genes to *Nicotiana tabacum* cells (see Materials and methods). A single cross-over event between the NPT II plasmid and homologous pBR322 sequences in pGV3850 introduces the NPT II chimaeric gene between the T-DNA borders of the pGV3850 Ti plasmid.

Several independent co-integrates from each NPT II plasmid were isolated and their structure confirmed by Southern blot analysis (data not shown). Three clones from each construction were used to transform regenerating protoplasts from *N. tabacum* Petit Havana cv. SR1 using a modified co-cultivation technique (Martón *et al.*, 1979). *Agrobacteria* were subsequently removed by the addition of the antibiotic, cefotaxim, to 500 μg/ml and the plant cells propagated with hormones for 2 weeks without selection. The micro-calli were then selected for resistance to Km at concentrations ranging from 50 μg/ml to 1000 μg/ml. Growth of resistant calli was observed as early as 3 weeks after the initiation of selection. Figure 4 shows the growth of resistant calli after the 4–6 weeks of selection at 1000 μg/ml Km.

The growth of resistant calli did not appear hindered at Km concentrations up to 1 mg/ml although a later attempt to propagate pAK1003 transformed cells in liquid culture with 3 mg/ml kanaycin resulted in only limited growth. Untransformed or control calli were all killed within 3 weeks even at 50 μg/ml Km. It was also noted that resistant calli in cultures containing higher densities of plant material grew somewhat slower under selection, possibly due to toxic products released by their dying neighbors (note pAK1003, Figure 4).

Enzymatic analysis of resistant tobacco calli

An enzymatic assay for neomycin phosphotransferase II has been developed by Reiss *et al.* (1984) and adapted to plant systems by P. Schreier (personal communciation). The NPT II enzyme is separated from contaminating phosphatases by native acrylamide gel electrophoresis prior to *in situ* phosphorylation of Km with radiolabeled γ-ATP. The ^{32}P-labeled Km is then bound to phosphocellulose paper and autoradiographed. Kanamycin-resistant calli from all three NPT II plasmids were tested and found to have considerable NPT II activity, compared with no detectable activity from

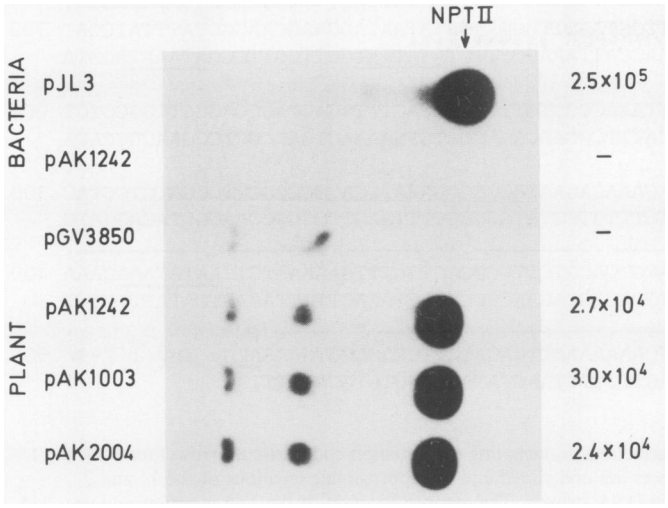

Fig. 5. Neomycin phosphotransferase II assay of resistant calli. The autoradiograph of ^{32}P phosphorylated kanamycin produced by electrophoretically separated NPT II enzyme (see Results section). The leftmost spots (gel top) result from non-specific phosphorylases and do not require Km as a substrate. Assays were made using crude extracts from both plant (resistant calli) and bacteria containing the indicated plasmid constructions. Due to higher specific activity, the exposure time for the bacterial lanes was ~1/10 that of the plant lanes. Radioactivity within the spots corresponding to the NPT II enzyme were scintillation counted and normalized to the total protein content of the individual extracts. These results are shown at the right. No NPT II activity was found within either the pGV3850 plant extracts or the pAK1242 bacterial extract.

either callus or plant tissue transformed with pGV3850 (Figure 5).

Introduction of the NPT II chimaeric genes into tobacco using co-cultivation results in numerous independent transformation events, each of which could potentially vary significantly in several factors effecting the level of expression of the inserted gene. Such factors include: the copy number of introduced T-DNA and the chromosomal environment of the integrated genes (DNA methylation, chromatin structure). Furthermore, adhesion of separate protoplasts during co-cultivation of callus growth could result in chimaeric calli, containing independently transformed plant cells (R. Hain, personal communication). Any quantitative comparison of the strength of the 1′ and 2′ promoters in the NPT II resistant calli must take such factors into consideration. The approach taken was to pool many independent calli from each co-cultivation and in this way average the impact of individual transformants on total enzyme levels. Assays of NPT II activity in tissue transformed with pAK1003 (1′-NPT II), pAK2004 (2′-NPT II) and pAK1242 (1′-NPT II) were made using a pool of ~50 individual calli, all selected in liquid media with 100 μg/ml Km. The results of liquid scintillation counting of the radiolabeled Km for each reaction are listed beside the appropriate lane in Figure 5. NPT II activity varied only ± 10% between calli pools from the three different TR-NPT II constructions, roughly the level of reproducibility of the enzymatic assay (data not shown). These data indicate that, at least under the conditions used (undifferentiated growth and Km selection), both 1′ and 2′ promoters result in approximately the same level of NPT II enzyme. Furthermore, insertion of the 2.2-kb Cm resistance fragment downstream from the 2′ promoter of pAK1003 (the pAK1242 plasmid) has no significant effect upon gene expression from the 1′-NPT II fusion.

Expression of the NPT II gene fused to both the 1′ (pAK1003) and 2′ (pAK2004) promoters was also compared with that from the NOS promoter-NPT II fusion of the pLGV1103 plasmid (Herrera-Estrella *et al.*, in preparation). NPT II assays were performed using extracts from several individual calli grown under 1000 μg/ml (data not shown). Enzyme levels were found to vary somewhat between individual calli transformed with the same chimaeric gene. However, comparison of the maximum observed activity from all three fusions (1′-NPT II, 2′-NPT II and pNOS-NPT II) suggested roughly the same efficiency of gene expression initiated by the three promoter regions.

Discussion

The most extensively analysed T-DNA promoters to date are those associated with the opine synthase genes, octopine and nopaline synthase. Limited 5′ deletion analysis has indicated that the minimum required sequence for the NOS promoter lies within 261 bp of the mRNA initiation site, while expression of the OCS gene requires a sequence >170 bp but <292 bp upstream of the transcriptional start (Koncz *et al.*, 1983). Examination of the transcript maps (Willmitzer *et al.*, 1982; Velten *et al.*, 1983; Winter *et al.*, 1984; Karcher *et al.*, 1984) and DNA sequence (Barker *et al.*, 1983; Gielen *et al.*, 1984) of the octopine T-DNA indicates that, with the possible exception of the 1′−2′ intergenic region of TL, the non-coding sequences upstream of T-DNA genes contain a minimum of 300 bp, consistent with the T-DNA promoter size of ~250 bp suggested by the OCS and NOS data. We have found that a 479-bp DNA fragment of TR-DNA, comprising all but 3 bp of the untranslated sequence between the divergently transcribed 1′ and 2′ genes, contains two intact promoters, both capable of expression when fused to the NPT II gene of Tn5. At roughly double the suggested size of the OCS and NOS promoters, the 1′−2′ promoter fragment would appear to contain the minimum sequence necessary for two adjacent T-DNA promoters.

There is currently little data concerning which sequences within the 1′−2′ intergenic region are important in controlling transcriptional initiation. Transcription in transformed plant tissue starts ~60 bp upstream from the initiation codons of both the 1′ and 2′ open reading frames (Winter *et al.*, 1984), ~30 bp downstream from possible 'TATA' consensus sequences (Figure 6). There are no obvious 'CAAT' or 'AGGA' (Messing *et al.*, 1983) consensus sequences upstream of either 'TATA' region. Transposon mutagenesis of the TR-DNA has produced two insertions mapping at the 1′−2′ promoter region (Figure 6), both of which effect the production of agropine and its precursors. The proposed pathway for agropine biosynthesis includes three steps, the first and second being catalysed by the products of the 2′ and 1′ genes respectively (Ellis *et al.*, 1984). Mutant number 14 maps to the 1′ half of the dual promoter fragment and, as expected, eliminates the production of the last two opines in the agropine pathway (Ellis *et al.*, 1984). The more interesting 2412 mutant maps within the 2′ half of the 1′−2′ intergenic region but, instead of abolishing all opine production, this mutation results in reduced levels of all the agropine type opines (Salomon *et al.*, 1984). These data suggest that sequences exist within the dual promoter fragment which, at least for the 2′ promoter, are not essential for gene expression but do modify the efficiency of transcription. Detailed *in*

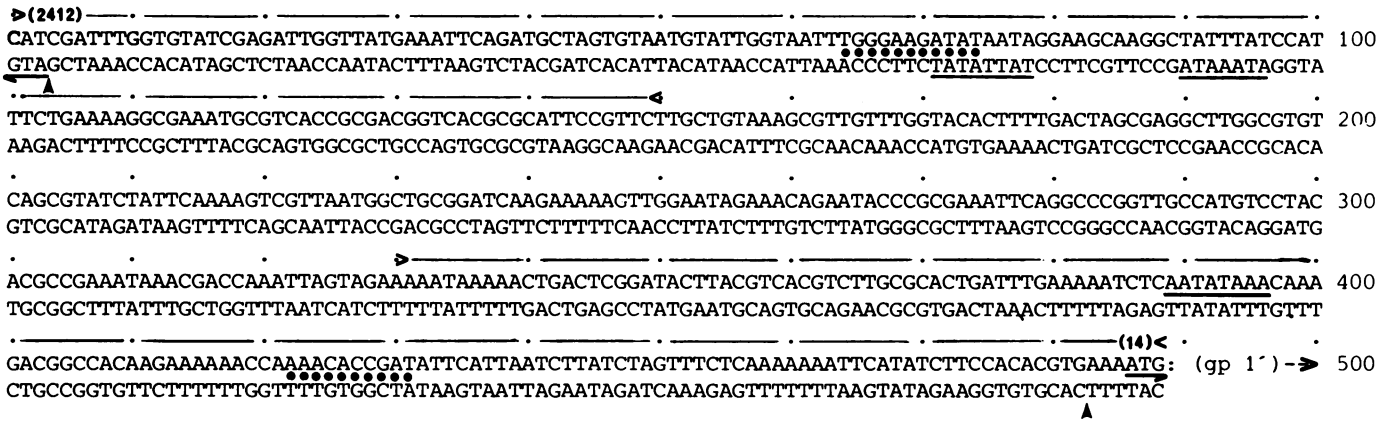

Fig. 6. DNA sequence of the 1′ and 2′ promoters. The DNA sequence of the intergenic region, including the initiation codons (half arrows) for both the 1′ (right end) and 2′ (left end) open reading frames, is listed. Potential 'TATA' sequences are underlined and the approximate locations of the 1′ and 2′ mRNA 5′ ends (Winter *et al.*, 1984) are indicated by two groups of dots between the DNA strands. The mapped location of the Tn5 insertion mutants #14 and #2412 (accuracy ±75 bp) are shown by lines above the listed sequence. The boundaries of the cloned promoter fragment are indicated by vertical arrows.

vitro mutagenesis, currently underway, should help locate such sequences, as well as any possible overlap of the two transcriptional control regions. Measurement of the effect of single mutations upon both promoters will be possible after fusion of a second easily assayed enzyme, chloramphenicol acetyl-transferase (Herrera-Estrella *et al.*, 1983b), to the promoter opposite the NPT II fusion (in progress).

The strength of both the 1′ and 2′ promoters were found to be essentially equivalent when measured at the level of gene product, NPT II, produced within resistant calli cultured under identical Km concentration (Figure 5). These NPT II assays were made using pools of ~50 microcalli since enzyme levels were found to vary significantly between individual calli (data not shown). It is known that transformation with *Agrobacterium* can result in multiple insertions of the T-DNA (Thomashow *et al.*, 1980; Zambryski *et al.*, 1983) and varying levels of gene expression between different tumor lines (Karcher *et al.*, 1984). It seems likely that both the copy number and the transcriptional environment at the locus of T-DNA insertion may effect the transcription of inserted genes. We are currently accumulating sufficient material from individual Km-resistant calli and tobacco plants to examine the molecular basis of the clonal variability of NPT II levels.

Transfer of either the 1′ or 2′-NPT II fusions into regenerating tobacco protoplasts allowed the direct and efficient selection of transformed cells by their resistance to Km in the media (see Figure 4). Similar selection for Km resistance has been reported in tobacco (Herrera-Estrella *et al.*, 1983a; Bevan *et al.*, 1983) and petunia (Fraley *et al.*, 1983) transformed with several independently derived fusions of the NOS promoter to NPT II. The dual promoter plasmids are unique, however, in that selection for Km resistance makes use of only one of the two plant promoters present, leaving the other available for the expression of a second gene. Thus, the plasmids described in this report are potential selection-expression vectors for plant systems.

The use of the TR promoters within a selection-expression vector system would have several important advantages. First, as indicated by the broad host range of crown gall disease (De Cleen and De Ley, 1976), the T-DNA promoters are likely to be active in many different species of dicotyledonous plants. The T-DNA promoters also appear to be relatively simple, constitutively expressed plant promoters with very little tissue specificity. These characteristics are essential for the expression of selectable markers of transformation and could be useful in certain applications of genetic engineering in plants. Second, the compact 479-bp fragment, containing two separate bidirectional promoters, would, when fused to both the NPT II and a second gene, ensure the closest possible linkage between the selectable marker (Km resistance) and the gene to be expressed. Such close linkage reduces the possible transfer and integration of the Km resistance marker without the gene of interest. Furthermore, since the two chimaeric genes would be directly adjacent at the 5′ ends, factors influencing the transcriptional efficiency of one promoter (such as integration site) are likely to similarly effect expression of the other gene. Thus, selection for Km resistance would indicate a high probability of expression of the second gene. By the same reasoning, use of the dual promoter fragment to express two separate genes of interest (e.g., two enzymes from a common biosynthetic pathway) would help to ensure concurrent expression of the two genes at roughly equivalent levels. We are currently making use of these vectors to introduce and express several prokaryotic and heterologous eukaryotic genes in plants. Further manipulations of the dual-promoter plasmids to increase their usefulness as selection-expression vectors is also underway.

Materials and methods

Bacterial strains and plant lines

The *Escherichia coli* strain HB101, DH1 and JM83 were used for DNA transformations and plasmid growth. Conjugation between *E. coli* and *A. tumefaciens* used the strain GJ23 (Van Haute *et al.*, 1983). *A. tumefaciens* strain C58C1(rif) (Zambryski *et al.*, 1983) containing the pGV3850 Ti plasmid was used for transformation of *N. tabacum* Petit Havana cv. SR1.

DNA preparation and manipulation

DNA for analysis was prepared by the method of Birnboim and Doly (1979) and a large-scale preparation used the methods of Maniatis *et al.* (1982). Enzymatic reactions (with restriction enzymes, exonuclease *Bal*31, T4 DNA ligase and Klenow fragment of DNA polymerase I) were performed as described in Maniatis *et al.* (1982). Electrophoretic purification of DNA fragments was as described by Koncz *et al.* (1983). Purification, electrophoresis and hybridization analysis of total *A. tumefaciens* was according to Dhaese *et al.* (1979). DNA was labeled using a nick-translation kit from BRL.

Media and culture

Bacterial media used were Luria broth (LB) and Minimal A (Min A) media from Miller (1972). Antibiotic concentrations used were (*E. coli; A. tume-*

faciens): ampicillin (100 μg/ml; --), carbenicillin (--; 100 μg/ml), kanamycin (25 μg/ml; 25 μg/ml), chloramphenicol (10 μg/ml; --) and rifampicin (--; 100 μg/ml).

Screening for β-galactosidase activity in the pUC vectors was on Luria plates containing 40 μg/ml X-gal and 160 μg/ml IPTG (Messing and Vieira, 1982).

Plant media used was K3 (Martón *et al.*, 1979), liquid, and Murashige and Skoog (MS), solid (Murashige and Skoog, 1962). Bacteria in the co-cultivations were killed with 500 μg/ml cefotaxim (Hoechst). Selection for Km-resistant calli was in K3 media containing between 50 and 1000 μg/ml kanamycin acid sulfate (Sigma). Tobacco calli were propagated at 28°C under low light (~500 lux).

DNA sequence analysis

The dideoxy chain termination method of Sanger (Sanger *et al.*, 1977) was used. Both [α-^{32}P]CTP and [α-^{35}S]ATP were used as radiolabel.

Transfer of chimaeric genes to plants

The Ti plasmid vector of Zambryski *et al.* (1983) was used to transform tobacco cells with the TR-NPT II chimaeric genes. *A. tumefaciens* containing the pGV3850 plasmid were mated with *E. coli* containing, as well as the appropriate NPT II-TR promoter fusion plasmid, two plasmids contributing the transfer and mobilization functions necessary for conjugative transfer of ColE1 type plasmids to *Agrobacterium* (Van Haute *et al.*, 1983). The pBR derived replicative origin of the NPT II plasmids does not function in *A. tumefaciens*, requiring co-integration of the pLGV1103 derived plasmids into the Ti plasmid for retention of the bacterial Km resistance determinant (from Tn930). Co-integration occurs *via* homologous recombination between the pBR322 sequences of the NPT II plasmids and a copy of pBR322 contained within the T-DNA of pGV3850. The pBR322 insert of pGV3850 replaces most of the T-DNA of pGV3839 (Joos *et al.*, 1983), a nopaline type Ti plasmid, removing the transforming functions and leaving both right and left border sequences and the NOS gene. Ti plasmid co-integrates were selected by resistance to both rifampicin and Km and their structures verified by Southern analysis of total *Agrobacterium* DNA.

Transformation of regenerating tobacco cells followed a modification of the co-cultivation procedure of Martón *et al.* (1979). Protoplasts from sterile shoot cultures of *N. tabacum* cv. Petit Havana SR1 (Maliga *et al.*, 1973) were isolated according to Nagy and Maliga (1976).

Protoplasts (~10^5/ml) were cultured in K3 media (0.4 M sucrose as osmoticum, supplemented with 0.1 mg/l naphthylacetic acid (NAA) and 0.2 mg/l kinetin) for 2 days in the dark and 1~2 days at low light (~500 lux). Regenerating protoplasts were then inoculated with 100–200 Agrobacteria (grown in Min A medium) per protoplast and both co-cultured for 3–4 days at 20°C. Plant cells were then washed twice with isotonic sea water and further cultured in K3 media containing 1 mg/l NAA, 0.2 mg/l kinetin and 500 μg/ml of the antibiotic cefotaxim (Hoechst). The plant cell suspensions were diluted each week with fresh medium and the osmotic pressure gradually reduced (0.05 M sucrose per week). Kanamycin selection of transformants was started 3 weeks after co-cultivation, in either agarose bead (Shillito *et al.*, 1983) or liquid culture. Resistant calli could be distinguished as early as 2–3 weeks after selection. Using this procedure, transformation frequencies of up to 40% of surviving protoplasts were obtained.

Enzymatic assay of neomycin phosphotransferase II

The *in situ* gel assay for NPT II activity developed by Reiss *et al.* (1984) was used with slight modifications for plant tissues (P. Schreier, personal communication). Due to a noted variability in the NPT II levels of individual calli transformed with the same chimaeric NPT II gene, pools of ~50 microcalli (cultured under identical conditions and selected for resistance to 100 μg/ml Km), were used to compare relative promoter strengths. Assay quantification involved removal and scintillation counting of the phosphorylated Km spots. The values reported in Figure 5 were normalized to total protein concentration of the crude extract determined according to Bradford (1976).

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